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(54) Title: VIRUS PROTEIN PURIFICATION FROM VIROSOMES

(57) Abstract

The invention provides purification methods and crystallized viral attachment protein (VAP) of virosomes derived from a virus, wherein (a) the crystallized VAP is suitable for x-ray crystallography analysis; (b) x-ray analysis provides diffraction patterns of sufficient resolution to determine the three-dimensional structure of the VAP; (c) the crystallized VAP is in biologically active form, as well as a specific crystallized VAP, hemagglutinin neuraminidase (HN) from a strain of Paramyxovirus, including nucleic acid, vectors and host cells having nucleotide sequences encoding the HN.

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Virus Protein Purification from Virosomes

Background of the Invention

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention utilized U.S. Government funds as NIH Grant No. 5R01AI11949-20. The U.S. Government has certain rights in this invention.

Field of the Invention

The present invention generally pertains to the field of viral protein crystallization. The present invention specifically pertains to crystallization methods and crystallized viral attachment proteins (VAPs), obtained from virosomes. The crystallized VAP is biologically active. Crystallized VAP, nucleic acid, vectors and host cells of a strain of paramyxovirus are also provided.

Related Art

VAP and Infection

Many infectious viruses contain an inner virion core having nucleic acid and a lipid envelope which holds the transmembrane (hydrophobic) domains of the envelope proteins. Some of the envelope proteins are viral attachment proteins (VAPs) that contain extracellular domains. Viruses infect a target cell by association of the virus' VAP with the target cell's viral receptor.

The extracellular domain of the VAP binds the target cell receptor and the transmembrane domain anchors the VAP to the viral envelope. (White *et al.*, *Quant. Rev. Biophys.* 16: 151-195 (1983)). After association of the VAP with the cell's viral receptor, the virion core enters the cytoplasm of the bound cell and the viral replication process is initiated. In some cases, viruses that are bound to target cell receptors can enter the cells by receptor mediated endocytosis.

Infectious Envelope-Containing Viruses

Examples of infectious envelope-containing viruses include, but are not limited to, togaviruses (yellow fever, RSSE and rubella); retroviruses (leukemia, sarcomas); orthomyxoviruses (influenza A, B, C); paramyxoviruses (mumps, measles, parainfluenza, Newcastle disease); rhabdoviruses (rabies); hepatitis virus; herpes viruses (herpes simplex, varicella zoster, cytomegalovirus, Epstein-Barr); and poxviruses (varcola, vaccinia, *Molluscum Contagiosum*). *See, e.g.*, Lycke and Norrby, eds. *Textbook of Medical Virology*, Chs. 1-4 and 7-9, Butterworths, London (1983).

Paramyxoviruses

Paramyxoviruses are one of three genera of the family *Paramyxoviridae*, which includes enveloped, negative-stranded RNA viruses. Paramyxoviruses utilize hemagglutinin neuraminidase (HN) as the target VAP (Fraenkel-Conrat and Wagner, eds., *Comprehensive Virology*, Vol. 4, Plenum Publishing Corp., New York (1975), pp. 99-178). Important examples of paramyxoviruses include mumps, measles, parainfluenza virus (PIV), Sendai virus (SV) and Newcastle disease virus (NDV).

Epidemiologically, particular strains of paramyxovirus are responsible for annual epidemics in humans. Reinfection by the same or similar strains in subsequent years is common, although less severe (Kass, ed., *Studies in Infectious Diseases Research*, The University of Chicago Press, Chicago (1975), pp. 51-64).

While some immunity develops through natural infection by most strains of paramyxoviruses, this immunity is generally not sufficient to provide complete protection. Although a few vaccines have been developed for some paramyxoviruses, these vaccines have limited effectiveness in the short term, and are generally ineffective in the long term (Choppin and Scheid, *Rev. Infect. Dis. 2*:40-61 (1980); Norrby *et al.*, *J. Infect. Dis. 132*:262-269 (1975)).

Structural Organization of the Paramyxoviruses

Electron micrographs have demonstrated that all paramyxovirus species have the same basic morphology. This morphology includes highly pleomorphic particles that are enclosed by a lipid envelope acquired during maturation. Maturation usually occurs by virus budding from the plasma membrane of the host cell.

The viral membrane of a paramyxovirus contains two virus-specified glycoproteins, HN and F. HN and F are found in all strains of paramyxoviruses. HN and F are attached to the viral membrane by short N-terminal and C-terminal transmembrane peptide sequences, respectively. The nucleotide sequence of HN genes of several paramyxoviruses has been determined. *See*, e.g., Gorman *et al.*, *Virology 175*:211-221 (1990); Merson *et al.*, *Virology 167*:97-105 (1988)); Blumberg *et al.*, *Cell 41*:269-278 (1985); Paterson *et al.*, *Proc. Natl. Acad. Sci. USA 81*:6706-6710 (1984); Hiebert *et al.*, *J. Virol. 53*:1-6 (1985); Hsu and Choppin, *Proc. Natl. Acad. Sci. USA 81*:7732-7736 (1984); Thompson *et al.*, *J. Virol. 62*:4653-4660 (1988).

Biological Activities of the Paramyxovirus

Paramyxovirus infection is initiated by the action of the two glycoproteins, HN and F (Kingsbury, *supra*, pp. 347-382; Fields, ed., *Virology*, Raven Press, New York (1985), pp. 1241-1253). HN protein from different paramyxovirus strains exhibit the same biological activities. These activities include hemagglutination (HA), cell binding, neuraminidase (NA) activity, and fusion promoting activities (Fraenkel-Conrat, *supra*, pp. 99-178 and 293-407; Kessler *et al.*, *J. Gen. Virol. 37*:547-556 (1977); Scheid and Choppin, *Virology 62*:125-133 (1974); Ebata *et al.*, *Virology 183*:437-441 (1991); Morrison *et al.*, *J. Virol. 65*:813-822 (1991); Hu *et al.*, *J. Virol. 66*:1528-1534 (1992); Tanabayashi *et al.*, *Virology 187*:801-804 (1992); Horvath *et al.*, *J. Virol. 66*:4564-4569 (1992)).

Hemagglutination activity is the capacity of a virus to absorb to erythrocytes and, as a result, cause the erythrocytes to aggregate (agglutinate). For example, in the paramyxoviruses a protein projecting from the virus membrane surface (HN) mediates the attachment to a sialic acid glyco-conjugate receptors on the erythrocyte surface. The hemagglutination reaction (HA) is an example

of a relatively simple, quick, convenient and semi-quantitative way of detecting, identifying, titrating viruses, detecting viral antibody and studying virus attachment. Cell-binding activity is the capacity of a virus to attach to a variety of infectible host cells. The HN protein of paramyxoviruses mediates the attachments to host cells, via a sialic acid-containing glyco-conjugate receptor. Neuraminidase activity is the enzyme catalyzed cleavage of the α-ketosidic linkage between terminal sialic acid and an adjacent sugar residue. The HN protein of paramyxoviruses possess neuraminidase activity. Fusion promoting activity is the capacity of paramyxovirus VAPs (*e.g.*, HN, H) to provide an essential function that allows the fusion (F) protein to directly mediate virus host-cell and cell-to-cell membrane fusion. (Fraenkel-Conrat, *supra*, pp. 99-178 and 293-407; Kessler *et al.*, *J. Gen. Virol. 37*:547-556 (1977); Scheid and Choppin, *Virology 62*:125-133 (1974); Ebata *et al.*, *Virology 183*:437-441 (1991); Morrison *et al.*, *J. Virol. 65*:813-822 (1991); Hu *et al.*, *J. Virol. 66*:1528-1534 (1992); Tanabayashi *et al.*, *Virology 187*:801-804 (1992); Horvath *et al.*, *J. Virol. 66*:4564-4569 (1992)).

Structural Separation of HN Biological Activities

The structural separation of the biological activities of the HN of paramyxoviruses has been partially demonstrated in such species as NDV, SV, PIV-1, and PIV-3. Attachment, neuraminidase and fusion promoting functions of both the NDV HN and the SV HN have been dissociated by binding with different MAbs. Additionally, specific mutations of HN cDNA resulted in expressed HN protein having one particular function inhibited, but not others. *See, e.g.,* Bousse *et al.*, *Virology 204*:506-514 (1994); Bishop & Compans, eds., *Nonsegmented Negative Strand Viruses*, Academic Press, Orlando, Florida (1984), pp. 345-350; Portner *et al.*, *Virology 158*:61-68 (1987); Iorio and Bratt, *J. Immunol. 133*:2115-2119 (1984); Iorio *et al.*, *J. Gen. Virol. 73*:1167-1176 (1992); Sergel *et al.*, *Virology 193*:717-726 (1993); Sergel *et al.*, *Virology 196*:831-834 (1993). Such results suggest that the active sites on HN for these biological activities are separate.

HN cell-binding, neuraminidase, and fusion promoting activities, essential for virus infection and spreading, are conserved among all or most strains of paramyxoviruses, as reflected in the high degree of sequence identity among these proteins. Therefore, the determined three-dimensional structure of an HN from a strain of paramyxovirus is useful for rational design of inhibitors to treat

infections of many or most paramyxoviruses and may be applicable to other members of *Paramyxoviridae* family. By analogy, the crystal structures of neuraminidases, from influenza virus, *Salmonella tryphimurium*, and *Vibrio cholerae* show similar three-dimensional structures (Crennell *et al.*, *Structure 2*:535-544 (1994); Crennell *et al.*, *Proc. Natl. Acad. Sci. USA 90*:9852-9856 (1993)).

Summary of the Invention

The present invention provides methods of purifying and crystallizing a viral attachment protein (VAP) from an envelope containing virus. The present invention also provides crystallized VAP which is soluble and biologically active.

The present invention also provides antibodies specific for the VAP and host cells that produce the antibody.

The present invention further provides nucleic acid molecules encoding the VAP, as well as nucleic acid probes specific for portions of the nucleic acid molecule. Also provided are vectors and host cells comprising the molecule.

The present invention also provides a crystallized HN protein from a strain of a species of a paramyxovirus.

The present invention also provides antibodies specific for the HN and host cells that produce the antibody.

The present invention further provides nucleic acid molecules encoding the HN, as well as nucleic acid probes specific for portions of the nucleic acid molecule. Also provided are vectors and host cells comprising the nucleic acid.

The present invention also provides a crystallized HN protein from the Kansas strain of a species of a paramyxovirus: Newcastle disease virus (NDV). This HN crystallized protein is suitable for x-ray diffraction analysis. The x-ray diffraction patterns obtained by this analysis provide coordinates of moderately high to high resolution. These coordinates are useful for three dimensional modeling of the HN protein. The three dimensional modeling programs use these coordinates and the amino acid sequence to generate secondary, tertiary and quaternary structures of the Kansas NDV HN.

The present invention also provides antibodies specific for the Kansas NDV HN and antibody expressing host cells.

The present invention further provides nucleic acid molecules encoding the Kansas NDV HN, as well as nucleic acid probes specific for portions of the nucleic acid molecule. Also provided are vectors and host cells comprising the nucleic acid.

Other objects of the invention will be apparent to one of ordinary skill in the art from the following detailed description and examples relating to the present invention.

Brief Description of the Figures

Figure 1. Results are shown from the non-reduced SDS-polyacrylamide gel electrophoresis of protease-cleaved NH protein from NDV. Lane 1 contains 20 μg of purified virus. Lane 2 contains an envelope fraction containing 5 μg of uncleaved HN. Lane 3 contains 10 μg of cleaved HN. The samples in the SDS loading buffer lacked reducing agent (1% B-mercaptoethanol) and were boiled for 2 minutes before loading. HN under reducing conditions migrated in a similar position. No disulfide linked oligomers were thus evident.

Figure 2. Results are shown from the crystallization of purified cleaved HN using the hanging drop-vapor diffusion method. 1.0 μl of cleaved HN solution (10 mg/ml) was mixed with an equal amount of precipitant and left to equilibrate over a reservoir of the same composition as the precipitant and deionized water. The reservoir solution was composed of 0.5 ml 20% (w/v) PEG 4000, 0.16 M ammonium sulfate, buffered with sodium acetate, pH 4.6.

Figure 3. A diffraction pattern is presented from a crystal of Kansas strain NDV cleaved HN using an X-ray source. The resolution was 3.5 Å at the edge of the pattern.

Figure 4. A diffraction pattern is presented from a crystal of cleaved HN using a more powerful X-ray beam than in Figure 3, produced in synchrotron storage rings. The resolution of the pattern was 2.6 Å at the edge. Data was collected using a crystal frozen at -175°C.

Figure 5. The nucleotide sequence of the Kansas strain of NDV is presented.

Figure 6. The deduced amino acid sequence of an HN of the Kansas strain of NDV is presented.

Detailed Description of the Preferred Embodiments

The present invention overcomes one or more deficiencies of the related background art, by providing methods for crystallizing a viral attachment protein (VAP) from virosomes, where the crystals diffract x-rays with high resolution of 1.5-3.9Å, such as 2.4-27Å.

The present invention thus includes methods of purifying and crystallizing a VAP from virosomes derived from a virus. The present invention also provides crystallized VAP by these methods which is soluble and biologically active.

The present invention, in a non-limiting example, provides methods of purifying and crystallizing hemagglutinin neuraminidase (HN) from a strain of a paramyxovirus using virosomes. The present invention also provides crystallized HN by these methods which is soluble and biologically active.

The present invention also provides biologically active VAPs. A non-limiting example is an HN from the Kansas strain of a species of a paramyxovirus, the Newcastle disease virus (NDV). The VAP is also provided as a crystallized protein.

Overview of VAP Purification and Crystallization Methods

In general, a VAP from a virus is isolated in soluble form (*e.g.*, lacking the transmembrane domains) by cleavage employing a protease applied to purified viruses or virosomes, as described herein. The resulting cleaved VAP is in sufficient purity and concentration (*e.g.*, a monomer or dimer) for crystallization. The cleaved VAP is then isolated and assayed for biological activity and for lack of aggregation (which interferes with crystallization). The purified and cleaved VAP preferably runs as a single band under reducing or nonreducing polyacrylamide gel electrophoresis (PAGE) (nonreducing is used to evaluate the presence of cysteine bridges).

The purified cleaved VAP is preferably crystallized using the hanging drop method under varying conditions of at least one of the following: pH, buffer type, buffer concentration, salt type,

polymer type, polymer concentration, other precipitating agents and concentration of purified and cleaved VAP. See, *e.g.*, the methods provided in a commercial kit, such as CRYSTAL SCREEN (Hampton Research, Riverside, CA). The crystallized protein is also tested for neuraminidase or cell binding biological activity and differently sized and shaped crystals are further tested for suitability for X-ray diffraction. Generally, larger crystals provide better crystallography than smaller crystals, and thicker crystals provide better crystallography than thinner crystals.

Virus Culture and Isolation Methods

To prepare isolated virus for purification of VAPs from virosomes, a strain of virus is diluted in a buffer solution at about neutral pH. The diluted virus solution can also be inoculated into the allantoic cavity of embryonated hen eggs for amplification. Tissue culture of a virus strain, or recombinant expression of the VAP can alternatively be used according to known method steps.

When allantoic culture is used, infected, embroynated eggs are incubated for several days and then chilled at about 4°C or less overnight. The allantoic fluids are collected and centrifuged at about 4°C or less to remove red blood cells. The virus in the supernatant is sedimented by ultracentrifugation at about 4°C or less. After the virus pellet is soaked in buffer solution overnight at about 4°C or less, the pellet is resuspended, *e.g.*, by homogenization.

The resuspended virus is optionally further purified by centrifugation in a sucrose gradient of about 5-50% at about 4°C or less. The sedimented virus is collected at a suitable sucrose percentage (*e.g.*, in the range of 5-50% sucrose), and sedimented again (after dilution with buffer) by ultracentrifugation at about 4°C or less. The sedimented, purified virus is then suspended in buffer containing suitable preservatives. *See, e.g.*, Portner *et al.*, *Virology 158*:61-68 (1987); Takimoto *et al.*, *J. Virol. 66*:7597-7600 (1992). The purified virus can then be used for virosome preparation.

Methods for Preparation of Purified Virosomes

To prepare HN or other VAP for crystallization, it is preferred that the protein be pure, in high concentration, biologically active, and/or have the transmembrane sequence removed.

Removal of the transmembrane domains is preferred since aggregation of the transmembrane or hydrophobic domains can inhibit crystallization. These objectives are alternatively accomplished by forming virosomes, when the purified virus itself cannot be suitably cleaved to provide cleaved VAP for crystallization.

Virosomes comprise reconstituted viral lipid envelope or liposome, containing surface viral proteins. The surface proteins have lipophilic or hydrophobic portions in the viral envelope or liposome, as well as extra cellular portions projecting from the envelope or liposome. The virosomes used in the present invention comprise a VAP such as hemagglutinin (HA), hemagglutinin neuraminidase (HN) or neuraminidase (NA), or other surface proteins that include, but are not limited to, F protein, sialidase, measles virus H protein, VSV G protein, gp120.

To form virosomes, purified virus is added to a proportional volume of a saline buffer containing a suitable detergent (*e.g.*, non-ionic detergent) to solubilize the virus. The mixture is then incubated at about room temperature with shaking. The preparation is then ultra-centrifuged at about 4°C or less to sediment the virus nucleocapsid and matrix proteins. The supernatant containing at least one type of VAP is collected and the detergent removed. The solution is then shaken at about room temperature or colder. Withdrawal of the detergent allows the virus membrane lipids and the virus envelope proteins to reform into a virosome as a lipid envelope containing the VAP extracellular portion projecting from the surface of the envelope. The solution is collected and the procedure repeated to substantially remove the detergent. The final solution contains the virosomes. *See, e.g.,* Almeida *et al., LANCET,* Nov. 8, 1975, 899-901.

The purified virosomes are optionally tested for biological activity (such as neuraminidase or sialidase activity) using known assays. *See, e.g.,* Aymard-Henry *et al., Bulletin of the World Health Organization, 48:*199-202 (1973); Thompson *et al., J. Virol. 62:*4653-4660 (1988); Takimoto *et al., J. Virol. 66:*7597-7600 (1992).

Viral Protein Purification Methods

Proteolytic cleavage by a protease is used to remove soluble portions of a VAP, from the transmembrane portion, contained in either the virus or the virosome. To a virosome solution is added a proteolytic enzyme (*e.g.*, pronase), and the mixture incubated at about room temperature

overnight. To remove the virosomes, the preparation is ultra-centrifuged at about 4°C or less. The cleaved VAP in the supernatant (as soluble protein) is collected and then optionally concentrated by further centrifugation.

The cleaved VAP is assayed for neuraminidase activity and for lack of aggregation, indicating that the transmembrane portion of the VAP has remained embedded in the virus envelope or virosome and is not part of the isolated protein. Removal of the hydrophobic membrane spanning region is preferred since aggregation of the hydrophobic regions can inhibit crystallization. After proteolytic treatment, virosomes and cleaved VAP are separated by centrifugation, with the cleaved VAP remaining in the supernatant. For example, a modification of a procedure described previously can be used (Thompson *et al.*, *J. Virol.* *62*:4653-4660 (1988)). The cleaved VAP fraction is preferably further concentrated by centrifugation through a filter, such as using a CENTRICON filter.

Purified Viral Proteins

The results of the purification are optionally analyzed by polyacrylamide gel electrophoresis (PAGE) under reducing or non-reducing conditions. A single band is preferably obtained. With disulfide-containing VAPs, it is preferred that the analysis of the cleaved VAP be under non-reducing conditions to indicate whether the cleaved protein formed disulfide linked dimers. The amino acid sequence can also be determined according to known methods, or otherwise obtained, as this sequence is important in determining the three dimensional structure of the cleaved protein (in combination with crystallographic analysis), as described herein, using molecular modeling techniques.

Before crystallization, biological activity (*e.g.*, neuraminidase (or sialidase) activity for HN, or other activity of the VAP) is determined using equivalent amounts of both virion- or virosome-associated VAP and purified and cleaved protein. It is preferred that the biological activity exceed the activity of the virion- or virosome-associated protein. The preferred result indicates that the cleaved protein retains its native structure, which is important for determining the three-dimensional crystal structure of the biologically active molecule. To identify the protease cleavage site, the purified and cleaved protein can be sequenced using known techniques. *See, e.g.*, Murti *et al.*, *Proc.*

Natl. Acad. Sci. USA 90:1523-1525 (1993); Takimoto *et al.*, *J. Virol. 66*:7597-7600 (1992), entirely incorporated herein by reference.

Viral Protein Crystallization Methods

The hanging drop method is preferably used to crystallize the cleaved protein. *See, e.g.*, Taylor *et al.*, *J. Mol. Biol. 226*:1287-1290 (1992); Takimoto *et al.*, *J. Virol. 66*:7597-7600 (1992); CRYSTAL SCREEN, Hampton Research.

A mixture of the cleaved protein and precipitant can include the following:

- pH (*e.g.*, 4-9);
- buffer type (*e.g.*, phosphate, sodium, or cacodylate acetates, imidazole, Tris HCl, sodium hepes);
- buffer concentration (*e.g.*, 10-200 mM);
- salt type (*e.g.*, calcium chloride, sodium citrate, magnesium chloride, ammonium acetate, ammonium sulfate, potassium phosphate, magnesium acetate, zinc acetate; calcium acetate)
- polymer type and concentration: (*e.g.*, polyethylene glycol (PEG) 1-50%, type 200-10,000);
- other precipitating agents (salts: K, Na, tartrate, NH_4SO_4, NaAc, $LiSO_4$, NaFormate, NaCitrate, MgFormate, $NaPO_4$, KPO_4 NH_4PO_4; organics: 2-propanol; non-volatile: 2-methyl-2,4-pentanediol); and
- concentration of purified cleaved VAP (*e.g.*, 5.0-100 mg/ml).

See, e.g., CRYSTAL SCREEN, Hampton Research.

A non-limiting example of such crystalization conditions is the following:

- purified cleaved protein (*e.g.*, 5-30 mg/ml);
- H_2O;
- precipitant 2-60% Polyethylene glycol (PEG) 500-5000 buffered with 10-200 mM phosphate or acetate buffer and 50-300 mM of a precipitating salt (*e.g.*, ammonium sulphate));
- at an overall pH of about 3.5-8.5.

The above mixtures are used and screened by varying at least one of pH, buffer type; buffer concentration, precipitating salt type or concentration, PEG type, PEG concentration, and cleaved protein concentration. Crystals ranging in size from 0.2-0.9 mm are formed in 1-14 days. These crystals diffract X-rays to at least 3.5 Å resolution, such as 1.5 -3.5 Å, or any range of value therein, such as 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, or 3.0, with 3.0 Å or less being preferred.

Viral Protein Crystals

Crystals appear after 1-14 days and continue to grow on subsequent days. Some of the crystals are removed, washed, and assayed for biological activity, which activity is preferred for using in further characterizations. Other washed crystals are preferably run on a stained gel and those that migrate in the same position as the purified cleaved VAP are preferably used. From two to one hundred crystals are observed in one drop and crystal forms can occur, such as, but not limited to, bipyramidal, rhomboid, and cubic. Initial X-ray analyses indicate that such crystals diffract at moderately high to high resolution, such as 1.5-3.5 Å or 2.2-2.7 Å. When fewer crystals are produced in a drop, they can be much larger size, *e.g.*, 0.4-0.9 mm.

Production and Use of Antibodies Specific to a VAP

The term "antibody", as used herein, refers both to monoclonal antibodies which are a substantially homogeneous population and to polyclonal antibodies which are heterogeneous populations. Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The term "antibody", as used herein, is also meant to include both intact molecules as well as fragments thereof, such as Fab and $F(ab')_2$, which are capable of binding antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and/or have less non-specific tissue binding than an intact antibody (Wahl *et al., J. Nucl. Med. 24*:316-325 (1983)). Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments). *See*, generally, Kohler and Milstein, Nature 256:495-497 (1975); U.S. Patent No. 4,376,110;

Ausubel *et al.*, eds., *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1987, 1992, 1993, 1994); and Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1988); Colligan *et al.*, eds., *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, N.Y., (1992, 1993), the contents of which references are entirely incorporated herein by reference.

Both monoclonal and polyclonal antibodies to a VAP, in crystalline or non-crystalline form, can be made according to methods well known in the art (*see*, e.g., Harlow, *supra*; Colligan, *supra*; Ausubel, *supra*, at §§11.4.2-11.13.4). Antibodies can be generated against VAP produced recombinantly or isolated from cells and tissues where the VAP is present, as in virally infected cells. Antibodies can be generated against the entire VAP or, more preferably, antibodies are generated against peptide subfragments representing functional domains of the VAP required for its cell binding activity, *e.g.*, the extracellular portion or a domain thereof. Antibodies for specifically inhibiting a VAP can be generated against peptide fragments unique to that protein. Alternatively, antibodies for generally inhibiting more than one member of a related class of VAPs can be generated against peptide fragments shared by the class of VAPs desired to be inhibited.

Cloning and Expression of Nucleic Acid Encoding a VAP

Known method steps for synthesizing oligonucleotides probes useful for cloning DNA encoding a VAP, such as an HN or HA, based on the teaching and guidance presented herein, are disclosed by, for example, in Ausubel, *infra*; Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); and Kaufman *et al., eds. Handbook of Molecular and Cellular Methods in Biology and Medicine*, CRC Press, Inc., Boca Raton (1995), which references are entirely incorporated herein by reference.

cDNA is generated from an envelope containing virus's RNA or virus-specific RNA from infected cells, or (in the case of DNA viruses) viral DNA is isolated, both from the virus and host cells containing the virus. A suitable oligonucleotide, or set of oligonucleotides, which is complementary to a sequence encoding a VAP is identified and hybridized to the DNA or cDNA. Single stranded oligonucleotide probes complementary to a unique portion of a VAP encoding sequence can be synthesized and labeled using known method steps. Such a probe can be used by

known procedures (or as a basis for synthesizing PCR probes) for amplifying DNA encoding a VAP from an envelope containing virus. Such oligonucleotide probes can be at least about 10 nucleotides in length (such as 10-30, 30-100, 100-500, or any range or value therein), in order to be specific for a target VAP encoding nucleic acid. Such procedures are well-known in the art. *See, e.g.,* Ausubel, *infra*, Sambrook, *infra*, and Kaufman, *infra*.

Culturing of the host and introduction of corresponding or complementary DNA or RNA into a vector and/or host cell can be performed by known methods. Any of a wide variety of vectors can be employed for this purpose. *See*, e.g., Ausubel, *infra*, §§ 1.5, 1.10, 7.1, 7.3, 8.1, 9.6, 9.7, 13.4, 16.2, 16.6, and 16.8-16.11. A nucleic acid sequence encoding a VAP of the present invention can be recombined with vector DNA in accordance with conventional techniques, *e.g.*, as disclosed by Ausubel, *infra*, Kaufman, *infra,* or Sambrook, *infra*. The vector is then incorporated into host cells (bacterial, yeast, insect or mammalian cells) using such vectors or viral vectors (*e.g.*, vaccinia, a retrovirus, an adenovirus or a baculovirus), according to known techniques.

Host cells comprising a nucleic acid which encodes a VAP of the present invention can be grown under conditions that provide expression of the VAP in recoverable or commercially useful amounts. *See*, e.g., Ausubel, *infra*, at §§ 1 and 13; Palese, U.S. Patent No. 5,166,057, which are entirely incorporated herein by reference.

Cloning of NDV HN cDNA

As a non-limiting example, cloning of NDV HN cDNA was performed by RT-PCR (reverse transcriptase polymerase chain reaction). Briefly, viral mRNA was isolated from virus infected mammalian cells and was then reverse transcribed into cDNA. The cDNA was subjected to PCR amplification using gene-specific (NDV HN specific) primers (corresponding to the DNA sequence presented in Figure 5. The amplified cDNA, which encodes NDV *HN* gene, was ligated into vector plasmid and then the plasmid was introduced into *E. coli*.

Virus (NDV) infected mammalian cells (BHK cells) were washed and suspended in a lysis buffer containing the nonionic detergent (Nonidet P-40). The intact nuclei were removed by a brief microfuge spin, and sodium dodecyl sulfate was added to the cytoplasm supernatant to denature protein. Protein was digested with protease and removed by extractions with phenol/chloroform and

chloroform. The cytoplasmic RNA which includes viral mRNA was recovered by ethanol precipitation. The isolated viral mRNA was used as a template to synthesize cDNA. First strand synthesis was driven by AMV reverse transcriptase and the oligo dT primer. Reverse transcriptases were derived from retroviruses such as avian myoblastosis virus (AMV) or Molony murine leukemia virus (MMLV), which use them to make DNA copies of their RNA genomes. Oligonucleotides were used as primers for extension on RNA templates. The DNA synthesized from the RNA template is complementary DNA (cDNA). PCR was used to amplify a segment of the cDNA. Two oligonucleotides were used as primers for a series of synthetic reactions that are catalyzed by a DNA polymerase (*e.g., Taq* DNA polymerase).

These oligonucleotides are complementary to sequences that (1) lie on opposite strands of the template DNA and (2) flank the segment of DNA that is to be amplified. These primers contain a potential restriction site at their 5′ termini to facilitate cloning of the amplified double-stranded cDNA into an appropriate vector. The major product of this reaction is a segment of double-stranded DNA whose termini are defined by the 5′ of the oligonucleotide primers and whose length is defined by the distance between the primers. The PCR product was cleaved with restriction enzyme which recognition sites were involved in the primers designed.

The NDV HN cDNA was then ligated into the plasmid vector pTF1 (Takahashi *et al., Genet. Anal. Tech. Appl. 9*:91-95 (1992)). The NDV HN cDNA was subcloned into HindIII and KpnI sites of the pTF1 vector. After litigation of vector DNA, the ligated DNA was introduced into *Escherichia coli (E. coli). E. coli* cells were transformed with the pTF1 vector containing the NDV HN cDNA using the calcium chloride precipitation method. The transfected cells were grown in nonselective medium to allow synthesis of plasmid-encoded antibiotic resistance protein, then plated on antibiotic-containing medium to allow identification of plasmid containing colonies. Positive transformants were selected using ampicillin containing medium for the ampicillin resistance gene in the pTF1 vector. Clones which included the plasmid pTF1 with NDV HN cDNA insert were isolated, grown in the ampicillin-containing medium and, after adding glycerol to 50%, stored at -70°C.

Having now generally described the invention, the same will be more readily understood through reference to the following example which is provided by way of illustration, and is not intended to be limiting of the present invention.

Example 1: Preparation, Purification and Crystallization of Hemagglutinin-Neuraminidase (HN) from a Paramyxovirus Using Virosomes

Propagation and purification of virus

Newcastle disease virus (Kansas strain) was diluted to 0.2 hemagglutination units (HA) in 10mM phosphate buffered saline (PBS pH7.4) containing gentamicin (0.5mg/ml: Bio Whittaker). Virus was inoculated into the allantoic cavity of 11-day-old embryonated hen eggs (0.1 ml/egg). The eggs were incubated at 35°C for two days and then chilled at 4°C overnight. The allantoic fluids were collected and centrifuged at 2,000 rpm for 30 min at 4°C in IEC CR-6000 centrifuge to remove red blood cells. The virus in the supernatant was sedimented by ultracentrifugation at 30,000 rpm for 1 hr at 4°C. After the virus pellet was soaked in PBS overnight at 4°C, the pellet was resuspended by homogenization in a dounce homogenizer. The resuspended virus was purified by centrifugation in a 30-50% sucrose gradient (PBS) at 27,000 rpm for 2 hrs at 4°C. The virus which sedimented at approximately 40% sucrose was collected and sedimented again, after adding at least 1.5 vol of PBS, by ultracentrifugation at 35,000 rpm for 1 hr at 4°C. The sedimented purified virus was suspended in PBS containing 0.1% sodium azide.

Forming virosomes by detergent withdrawal method

To prepare HN for crystallization, it is important that the HN be pure, in high concentration, biologically active, and have the transmembrane sequence removed. By forming virosomes these objectives were unexpectedly accomplished. To purified virus (20mg/ml), an equal volume of PBS containing 2% Triton X-100 (Sigma) was added to solubilize the virus. The mixture was then incubated at room temperature for 1 hr with gentle shaking. The preparation was next centrifuged at 35,000 rpm for 2 hrs at 4°C to sediment the virus nucleocapsid and matrix proteins. The

supernatant containing HN and F proteins was collected and Bio-Beads (Bio-Rad) (1 gram/5 ml supernatant) added to remove the detergent. The solution was gently shaken at room temperature for 1 hr. Withdrawal of the detergent allows the virus membrane lipids and the virus envelope proteins, HN and F, to reform into an envelope containing HN and F spikes projecting from the surface of the envelope. The solution was collected by syringe with a 27G needle. The procedure was repeated twice more to remove the detergent completely. The final solution contained the purified virosomes.

Isolation and purification of HN protein

Proteolytic cleavage with a protease was used to remove HN from the virosome. To 4 volumes of virosome solution (1.5mg/ml), 1 volume of pronase (0.5mg/ml in PBS)(CALBIOCHEM) was added and the mixture incubated at room temperature overnight. To collect the virosomes, the preparation was then centrifuged at 35,000 rpm for 1.5 hrs at 4°C. The cleaved HN protein in the supernatant was concentrated by centrifugation through CENTRICON-100 (AMICON) filter tubes. The concentrated HN was used for crystallization.

Figure 1, lane 3, shows the results of the purification, analyzed by polyacrylimide/gel electrophoresis (PAGE) under non-reducing conditions. A single band was obtained. Analysis of the HN under non-reducing conditions indicated that this HN of the Kansas strain of NDV did not form disulfide linked dimers. This strain is similar to the LaSota NDV strain which also does not show oligomeric HN under non-reducing PAGE analysis (Mirza *et al.*, *J. Biol. Chem. 268*:21425-21431 (1993)). The lack of cysteine in position 123 of the Kansas and LaSota strains (which is thought to be involved in disulfide bond formation) is likely responsible for the monomeric HN seen in the non-reducing gels. HN of this strain forms non-disulfide linked oligomers which are unstable under PAGE conditions. Additional characterization of the Kansas HN showed a protein migration pattern typical of NDV. We cloned and sequenced the Kansas HN gene (See Example 3) (Fig. 5) which showed a typical NDV HN sequence with up to 99% identity to HN from other NDV strains in the GenBank database. This sequence information is important in determining the three dimensional structure of HN from crystallographic analysis.

Before crystallization was undertaken, we measured the neuraminidase activity in equivalent amounts of virion associated HN and purified cleaved HN and found that cleaved HN activity was equivalent with the activity of the virion-associated HN (Table 3). This indicated that the cleaved HN retained its native structure, which is important for determining the three-dimensional crystal structure of a biologically active molecule. To identify the protease cleavage site, the purified cleaved HN was sequenced using automated Edman degradation. An exact match of 15 amino acids at the newly created amino terminus of the cleaved HN identified the cleavage site at Gly 124 ("†" in Figure 6).

Table 3

Neuraminidase Activity of NDV HN Purified From Virosomes or on Virus Particles		
Equivalent Amount of HN Protein (μg)	**Activity of HN Purified from Virus Particle(A^{549})**	**Activity HN Purified from Virosomes (A^{549})**
1.5	0.353	0.265
3.0	0.961	0.949

Neuraminidase activity of equivalent amounts of HN, comparing the native viral activity with cleaved and purified HN. The equivalent amounts of HN were incubated for 30 min at 37°C with 2.0 mg of N-acetylneuramin-lactose and then assayed for free sialic acid. HN represents ~25% of total virion protein.

The data in this table shows that purified HN is recovered, with no loss of biological activity in the cleaved purified monomers.

Crystallization of Cleaved HN

The hanging drop method was used to crystallize the cleaved HN protein.

A mixture of the cleaved HN protein and precipitant was made, as described below, to be dropped on the crystallization surface.

One volume of purified cleaved HN protein (10mg/ml)

- One volume of H_2O
- One volume of precipitant (20% Polyethylene glycol 4000 + 160mM $(NH_4)_2SO_4$ + 80mM Acetate Buffer pH 4.6)

The drops were made over a well containing the precipitate. Crystals ranging in size from 0.2-0.7 mm were formed in 2-7 days (Fig. 2). Some of these crystals were removed, washed, and assayed for neuraminidase activity, which they were found to retain. Other washed crystals were run on a stained gel and found to migrate in the same position as the cleaved HN. As many as 40 crystals were observed in one drop and a number of different crystal forms were noted, including bipyramidal, rhomboid, and cubic. Initial X-ray analyses discussed in the next section indicates that the 0.2-0.25 mm crystal diffracts at moderately high resolution. Fewer crystals were also produced in a drop, but of much larger size, 0.4-0.6 mm.

Example 2: X-ray Diffraction Analysis

The first crystals produced (~0.2-0.25 mm) were X-ray analyzed on a rotating Cu anode X-ray source operating at 40 kV and 100 mA.

Figure 3 shows the diffraction pattern from a single frame of several hundred collected. Crystals were stable for at least 20 hrs. Frozen crystals were used for longer X-ray exposures (48 hrs), the crystals being stable to the X-rays in the frozen state. To collect the maximum number of useful reflections, multiple frames were collected as the crystal was rotated in the X-ray beam for 48 hrs. In this analysis, crystals diffracted to a resolution of 3.5 Å (Fig. 4, edge). To increase the resolution further, slightly larger crystals (0.25 mm) were analyzed in a synchrotron high energy X-ray source. Using frozen crystals, X-ray diffraction data was collected every 6 minutes over a 24-hr period. A single frame is shown in Figure 5. The crystals diffracted to a relatively high resolution of 2.6 Å.

Example 3: Cloning and Sequencing of Nucleic Acid Encoding a Paramyxovirus HN Protein

HN gene of NDV was cloned by polymerase chain reaction (PCR) method using RNA extracted from virus infected BHK cells. First strand cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (Promega) using primer designed from consensus sequence found in NDV strains obtained from NIH GenBank. The synthesized cDNA was amplified by PCR using similarly designed primers. NDV *HN* cDNA containing full coding region was subcloned into plasmid pTF1 (Takahashi *et al.*, 1992, Bousse *et al.*, 1994) at HindIII and KpnI sites.

Sequencing of the NDV HN cloned in pTF1 was done by the dideoxy chain termination method using SEQUENASE version 2 DNA polymerase (US Biomedicals) following the manufacturers instructions. The primers used for sequence were designed from the sequence data of other NDV strains in NIH GenBank. The cDNA sequence is presented in Figure 4, and the corresponding amino acid sequence is presented in Figure 5. *See, e.g.,* Takahashi *et al., Genet. Anal. Tech. Appl. 9:*91-95 (1992); Bousee *et al., Virology 204:*506-514.

All references cited herein are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: St. Jude Children's Research Hospital
332 North Lauderdale
Memphis, TN 38105-2794
United States of America

(ii) TITLE OF INVENTION: Virus Protein Purification from Virosomes

(iii) NUMBER OF SEQUENCES: 2

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(D) STATE: D.C.
(E) COUNTRY: USA
(F) ZIP: 20005-3934

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: To be assigned
(B) FILING DATE: Herewith
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA
(A) APPLICATION NUMBER: U.S. 60/003,447
(B) FILING DATE: 08-SEP-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 30,353
(C) REFERENCE/DOCKET NUMBER: 0656.054PC01

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (202) 371-2600
(B) TELEFAX: (202) 371-2540

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1734 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both

(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1731

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG GAC CGC GCA GTT AGC CAA GTT GCG TTA GAG AAT GAT GAA AGA GAG      48
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 1               5                  10                  15

GCA AAA AAT ACA TGG CGC TTG ATA TTC CGG ATT GCA ATC TTA CTC TTA      96
Ala Lys Asn Thr Trp Arg Leu Ile Phe Arg Ile Ala Ile Leu Leu Leu
            20                  25                  30

ACA GTA GTG ACC TTA GCT ACA TCT GTA GCC TCC CTT GTA TAT AGC ATG     144
Thr Val Val Thr Leu Ala Thr Ser Val Ala Ser Leu Val Tyr Ser Met
        35                  40                  45

GGG GCT AGC ACA CCT AGC GAC CTT GTA GGC ATA CCG ACC AGG ATT TCT     192
Gly Ala Ser Thr Pro Ser Asp Leu Val Gly Ile Pro Thr Arg Ile Ser
    50                  55                  60

AGG GCA GAA GAA AAG ATT ACA TCT GCA CTT GGT TCC AAT CAA GAT GTA     240
Arg Ala Glu Glu Lys Ile Thr Ser Ala Leu Gly Ser Asn Gln Asp Val
65                  70                  75                  80

GTA GAT AGG ATA TAT AAG CAA GTG GCC CTT GAG TCT CCG TTG GCA TTG     288
Val Asp Arg Ile Tyr Lys Gln Val Ala Leu Glu Ser Pro Leu Ala Leu
                85                  90                  95

TTA AAC ACT GAG ACC ACA ATT ATG AAC GCA ATA ACA TCT CTC TCT TAT     336
Leu Asn Thr Glu Thr Thr Ile Met Asn Ala Ile Thr Ser Leu Ser Tyr
            100                 105                 110

CAG ATT AAT GGA GCT GCG AAC AAC AGC GGG TGG GGG GCA CCT ATC CAT     384
Gln Ile Asn Gly Ala Ala Asn Asn Ser Gly Trp Gly Ala Pro Ile His
        115                 120                 125

GAC CCA GAT TTT ATC GGG GGG ATA GGC AAA GAA CTC GTT GTA GAT AAT     432
Asp Pro Asp Phe Ile Gly Gly Ile Gly Lys Glu Leu Val Val Asp Asn
    130                 135                 140

GCT AGT GAT GTC ACA TCA TTC TAT CCC TCT GCA TTT CAA GAA CAT CTG     480
Ala Ser Asp Val Thr Ser Phe Tyr Pro Ser Ala Phe Gln Glu His Leu
145                 150                 155                 160

AAT TTT ATC CCG GCG CCT ACT ACA GGA TCA GGT TGC ACT CGG ATA CCT     528
Asn Phe Ile Pro Ala Pro Thr Thr Gly Ser Gly Cys Thr Arg Ile Pro
                165                 170                 175
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TCA TTT GAC ATG AGT GCT ACC CAT TAC TGC TAC ACT CAT AAT GTA ATA        576
Ser Phe Asp Met Ser Ala Thr His Tyr Cys Tyr Thr His Asn Val Ile
            180                 185                 190

TTG TCT GGA TGC AGA GAT CAC TCA CAC TCA CAT CAG TAT TTA GCA CTT        624
Leu Ser Gly Cys Arg Asp His Ser His Ser His Gln Tyr Leu Ala Leu
        195                 200                 205

GGT GTG CTC CGG ACA ACT GCA ACA GGG AGG ATA TTC TTT TCT ACT CTG        672
Gly Val Leu Arg Thr Thr Ala Thr Gly Arg Ile Phe Phe Ser Thr Leu
    210                 215                 220

CGT TCC ATC AGT CTG GAT GAC ACC CAA AAT CGG AAG TCT TGC AGT GTG        720
Arg Ser Ile Ser Leu Asp Asp Thr Gln Asn Arg Lys Ser Cys Ser Val
225                 230                 235                 240

AGT GCA ACT CCC TTA GGT TGT GAT ATG CTG TGC TCG AAA GTC ACG GAG        768
Ser Ala Thr Pro Leu Gly Cys Asp Met Leu Cys Ser Lys Val Thr Glu
                245                 250                 255

ACA GAG GAA GAA GAT TAT AAC TCA GCT GTC CCT ACG CTG ATG GCA CAT        816
Thr Glu Glu Glu Asp Tyr Asn Ser Ala Val Pro Thr Leu Met Ala His
            260                 265                 270

GGG AGG TTA GGG TTC GAC GGC CAA TAC CAC GAA AAG GAC CTA GAC GTC        864
Gly Arg Leu Gly Phe Asp Gly Gln Tyr His Glu Lys Asp Leu Asp Val
        275                 280                 285

ACA ACA TTA TTT GAG GAC TGG GTG GCC AAC TAC CCA GGA GTA GGG GGT        912
Thr Thr Leu Phe Glu Asp Trp Val Ala Asn Tyr Pro Gly Val Gly Gly
    290                 295                 300

GGA TCT TTT ATT GAC GGC CGC GTA TGG TTC TCA GTC TAC GGA GGG CTG        960
Gly Ser Phe Ile Asp Gly Arg Val Trp Phe Ser Val Tyr Gly Gly Leu
305                 310                 315                 320

AAA CCC AAT TCA CCC AGT GAC ACT GTA CAG GAA GGG AAA TAC GTA ATA       1008
Lys Pro Asn Ser Pro Ser Asp Thr Val Gln Glu Gly Lys Tyr Val Ile
                325                 330                 335

TAC AAG CGA TAC AAT GAC ACA TGC CCA GAT GAG CAA GAC TAC CAG ATC       1056
Tyr Lys Arg Tyr Asn Asp Thr Cys Pro Asp Glu Gln Asp Tyr Gln Ile
            340                 345                 350

CGA ATG GCC AAG TCT TCG TAT AAG CCC GGG CGG TTT GGT GGG AAA CGC       1104
Arg Met Ala Lys Ser Ser Tyr Lys Pro Gly Arg Phe Gly Gly Lys Arg
        355                 360                 365

ATA CAG CAG GCT ATC TTA TCT ATC AAG GTG TCA ACA TCT TTG GGC GAA       1152
Ile Gln Gln Ala Ile Leu Ser Ile Lys Val Ser Thr Ser Leu Gly Glu
    370                 375                 380

GAC CCA GCA CTG ACT GTA CCG CCC AAC ACA GTC ACA CTC ATG GGG GCC       1200
Asp Pro Ala Leu Thr Val Pro Pro Asn Thr Val Thr Leu Met Gly Ala
385                 390                 395                 400
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GAA GGA AGA ATT CTC ACA GTA GGG ACA TCT CAT TTC TTG TAT CAG CGA      1248
Glu Gly Arg Ile Leu Thr Val Gly Thr Ser His Phe Leu Tyr Gln Arg
                405                 410                 415

GGG TCA TCA TAC TTC TCT CCC GCG TTA TTA TAT CCT ATG ACA GTC AGC      1296
Gly Ser Ser Tyr Phe Ser Pro Ala Leu Leu Tyr Pro Met Thr Val Ser
            420                 425                 430

AAC AAA ACA GCC ACT CTT CAT AGT CCC TAT ACA TTC AAT GCC TTC ACT      1344
Asn Lys Thr Ala Thr Leu His Ser Pro Tyr Thr Phe Asn Ala Phe Thr
        435                 440                 445

CGG CCA GGT AGT ATC CCT TGC CAG GCT TCA GCA AGA TGC CCC AAC TCG      1392
Arg Pro Gly Ser Ile Pro Cys Gln Ala Ser Ala Arg Cys Pro Asn Ser
    450                 455                 460

TGT GTT ACT GGA GTC TAT ACA GAT CCA TAT CCC CTA ATC TTC TAT AGG      1440
Cys Val Thr Gly Val Tyr Thr Asp Pro Tyr Pro Leu Ile Phe Tyr Arg
465                 470                 475                 480

AAC CAC ACC TTG CGA GGG GTA TTC GGG ACA ATG CTT GAT AGT GAA CAA      1488
Asn His Thr Leu Arg Gly Val Phe Gly Thr Met Leu Asp Ser Glu Gln
                485                 490                 495

GCA AGA CTT AAT CCT GCG TCT GCA GTA TTC GAT AGC ACA TCC CGC AGT      1536
Ala Arg Leu Asn Pro Ala Ser Ala Val Phe Asp Ser Thr Ser Arg Ser
            500                 505                 510

CGC ATA ACT CGA GTG AGT TCA AGC AGC ACC AAA GCA GCA TAC ACA ACA      1584
Arg Ile Thr Arg Val Ser Ser Ser Ser Thr Lys Ala Ala Tyr Thr Thr
        515                 520                 525

TCA ACT TGT TTT AAA GTT GTC AAG ACC AAT AAG ACC TAT TGT CTC AGC      1632
Ser Thr Cys Phe Lys Val Val Lys Thr Asn Lys Thr Tyr Cys Leu Ser
    530                 535                 540

ATT GCT GAA ATA TCT AAT ACT CTC TTC GGA GAA TTC AGA ATC GTC CCG      1680
Ile Ala Glu Ile Ser Asn Thr Leu Phe Gly Glu Phe Arg Ile Val Pro
545                 550                 555                 560

TTA CTA GTT GAG ATC CTC AAA AAT GAT GGG GTT AGA GAA GCC AGG TCT      1728
Leu Leu Val Glu Ile Leu Lys Asn Asp Gly Val Arg Glu Ala Arg Ser
                565                 570                 575

GGT TAG                                                             1734
Gly
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 577 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Asp Arg Ala Val Ser Gln Val Ala Leu Glu Asn Asp Glu Arg Glu
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Ala Lys Asn Thr Trp Arg Leu Ile Phe Arg Ile Ala Ile Leu Leu Leu
            20                  25                  30

Thr Val Val Thr Leu Ala Thr Ser Val Ala Ser Leu Val Tyr Ser Met
        35                  40                  45

Gly Ala Ser Thr Pro Ser Asp Leu Val Gly Ile Pro Thr Arg Ile Ser
    50                  55                  60

Arg Ala Glu Glu Lys Ile Thr Ser Ala Leu Gly Ser Asn Gln Asp Val
65                  70                  75                  80

Val Asp Arg Ile Tyr Lys Gln Val Ala Leu Glu Ser Pro Leu Ala Leu
                85                  90                  95

Leu Asn Thr Glu Thr Thr Ile Met Asn Ala Ile Thr Ser Leu Ser Tyr
            100                 105                 110

Gln Ile Asn Gly Ala Ala Asn Asn Ser Gly Trp Gly Ala Pro Ile His
        115                 120                 125

Asp Pro Asp Phe Ile Gly Gly Ile Gly Lys Glu Leu Val Val Asp Asn
    130                 135                 140

Ala Ser Asp Val Thr Ser Phe Tyr Pro Ser Ala Phe Gln Glu His Leu
145                 150                 155                 160

Asn Phe Ile Pro Ala Pro Thr Thr Gly Ser Gly Cys Thr Arg Ile Pro
                165                 170                 175

Ser Phe Asp Met Ser Ala Thr His Tyr Cys Tyr Thr His Asn Val Ile
            180                 185                 190

Leu Ser Gly Cys Arg Asp His Ser His Ser His Gln Tyr Leu Ala Leu
        195                 200                 205

Gly Val Leu Arg Thr Thr Ala Thr Gly Arg Ile Phe Phe Ser Thr Leu
    210                 215                 220

Arg Ser Ile Ser Leu Asp Asp Thr Gln Asn Arg Lys Ser Cys Ser Val
225                 230                 235                 240

Ser Ala Thr Pro Leu Gly Cys Asp Met Leu Cys Ser Lys Val Thr Glu
                245                 250                 255

Thr Glu Glu Glu Asp Tyr Asn Ser Ala Val Pro Thr Leu Met Ala His
            260                 265                 270
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Gly Arg Leu Gly Phe Asp Gly Gln Tyr His Glu Lys Asp Leu Asp Val
275 280 285

Thr Thr Leu Phe Glu Asp Trp Val Ala Asn Tyr Pro Gly Val Gly Gly
290 295 300

Gly Ser Phe Ile Asp Gly Arg Val Trp Phe Ser Val Tyr Gly Gly Leu
305 310 315 320

Lys Pro Asn Ser Pro Ser Asp Thr Val Gln Glu Gly Lys Tyr Val Ile
325 330 335

Tyr Lys Arg Tyr Asn Asp Thr Cys Pro Asp Glu Gln Asp Tyr Gln Ile
340 345 350

Arg Met Ala Lys Ser Ser Tyr Lys Pro Gly Arg Phe Gly Gly Lys Arg
355 360 365

Ile Gln Gln Ala Ile Leu Ser Ile Lys Val Ser Thr Ser Leu Gly Glu
370 375 380

Asp Pro Ala Leu Thr Val Pro Pro Asn Thr Val Thr Leu Met Gly Ala
385 390 395 400

Glu Gly Arg Ile Leu Thr Val Gly Thr Ser His Phe Leu Tyr Gln Arg
405 410 415

Gly Ser Ser Tyr Phe Ser Pro Ala Leu Leu Tyr Pro Met Thr Val Ser
420 425 430

Asn Lys Thr Ala Thr Leu His Ser Pro Tyr Thr Phe Asn Ala Phe Thr
435 440 445

Arg Pro Gly Ser Ile Pro Cys Gln Ala Ser Ala Arg Cys Pro Asn Ser
450 455 460

Cys Val Thr Gly Val Tyr Thr Asp Pro Tyr Pro Leu Ile Phe Tyr Arg
465 470 475 480

Asn His Thr Leu Arg Gly Val Phe Gly Thr Met Leu Asp Ser Glu Gln
485 490 495

Ala Arg Leu Asn Pro Ala Ser Ala Val Phe Asp Ser Thr Ser Arg Ser
500 505 510

Arg Ile Thr Arg Val Ser Ser Ser Ser Thr Lys Ala Ala Tyr Thr Thr
515 520 525

Ser Thr Cys Phe Lys Val Val Lys Thr Asn Lys Thr Tyr Cys Leu Ser
530 535 540

Ile Ala Glu Ile Ser Asn Thr Leu Phe Gly Glu Phe Arg Ile Val Pro
545 550 555 560

Leu Leu Val Glu Ile Leu Lys Asn Asp Gly Val Arg Glu Ala Arg Ser

565 570 575

Gly

What Is Claimed Is:

1. A method for crystallizing a viral attachment protein (VAP) from a virus, comprising

(a) providing a purified virosome derived from said virus containing the VAP in membrane bound form;

(b) cleaving, with a proteolytic enzyme, the soluble portion of the VAP from the transmembrane portion of the VAP in said virosome, to provide a soluble form of the VAP as a cleaved VAP having biological activity; and

(c) crystallizing the cleaved VAP using a hanging drop vapor diffusion method, to provide crystallized cleaved VAP having biological activity.

2. A method according to claim 1, wherein said proteolytic enzyme is selected from the group consisting of pronase, trypsin, thermolysin, protease K, pronase, papain, endoproteinase Lys-c, endoproteinase Gly-c, endoproteinase Asp-N, endoproteinase Arg-c, chymotrypsin, bromelain, carboxypeptidase y, carboxypeptidase P, carboxypeptidase A, carboxypeptidase B, aminopeptidase M, pepsin, plasmin, and leucine aminopeptidase

3. A method according to claim 1, wherein said VAP is hemagglutinin neuraminidase (HN).

4. A method according to claim 1, wherein said crystallization step is done under conditions of 5-100 mg/ml cleaved VAP; PEG100-8000; precipitating salt; buffered saline, and pH 4-9.

5. A method according to claim 1, wherein said virus strain is of a paramyxovirus.

6. A method according to claim 5, wherein said paramyxovirus is selected from mumps, measles, parainfluenza virus (PIV), sendai virus (SV) and Newcastle disease virus (NDV).

7. A method according to claim 6, wherein the strain of the paramyxovirus is the Kansas strain of Newcastle disease virus (NDV).

8. A method according to claim 7, wherein the crystallization conditions are one volume of 5-00 mg/ml of cleaved HN; one volume of water; and one volume of 20-25% PEG3350-4000, 160-200 mM ammonium sulfate and 80-100 mM acetate buffered saline (pH 4.2-4.8).

9. A method according to claim 1, wherein the cleaved VAP crystals have biological activity and provide a wider x-ray crystallograph of about 5-3.5 Å.

10. A method according to claim 9, wherein the resolution is 2.6 Å.

11. A crystallized cleaved VAP , provided by a method according to claim 1.

12. A crystallized cleaved VAP according to claim 11, wherein the crystals are of an HN of the Kansas strain of NDV.

13. A paramyxovirus HN protein, comprising the amino acid sequence:

APIHDP DFIGGIGKEL VVDNASDVTS
FYPSAFQEHL NFIPAPTTGS GCTRIPSFDM SATHYCYTHN VILSGCRDHS
HSHQYLALGV LRTTATGRIF FSTLRSISLD DTQNRKSCSV SATPLGCDML
CSKVTETEEE DYNSAVPTLM AHGRLGFDGQ YHEKDLDVTT LFEDWVANYP
GVGGGSFIDG RVWFSVYGGL KPNSPSDTVQ EGKYVIYKRY NDTCPDEQDY
QIRMAKSSYK PGRFGGKRIQ QAILSIKVST SLGEDPALTV PPNTVTLMGA
EGRILTVGTS HFLYQRGSSY FSPALLYPMT VSNKTATLHS PYTFNAFTRP
GSIPCQASAR CPNSCVTGVY TDPYPLIFYR NHTLRGVFGT MLDSEQARLN
PASAVFDSTS RSRITRVSSS STKAAYTTST CFKVVKTNKT YCLSIAEISN
TLFGEFRIVP LLVEILKNDG VREARSG

14. A paramyxovirus HN protein according to claim 13, comprising the amino acid sequence:

```
  1  MDRAVSQVAL ENDEREAKNT WRLIFRIAIL LLTVVTLATS VASLVYSMGA
 51  STPSDLVGIP TRISRAEEKI TSALGSNQDV VDRIYKQVAL ESPLALLNTE
101  TTIMNAITSL SYQINGAANN SGWGAPIHDP DFIGGIGKEL VVDNASDVTS
151  FYPSAFQEHL NFIPAPTTGS GCTRIPSFDM SATHYCYTHN VILSGCRDHS
201  HSHQYLALGV LRTTATGRIF FSTLRSISLD DTQNRKSCSV SATPLGCDML
251  CSKVTETEEE DYNSAVPTLM AHGRLGFDGQ YHEKDLDVTT LFEDWVANYP
301  GVGGGSFIDG RVWFSVYGGL KPNSPSDTVQ EGKYVIYKRY NDTCPDEQDY
351  QIRMAKSSYK PGRFGGKRIQ QAILSIKVST SLGEDPALTV PPNTVTLMGA
401  EGRILTVGTS HFLYQRGSSY FSPALLYPMT VSNKTATLHS PYTFNAFTRP
451  GSIPCQASAR CPNSCVTGVY TDPYPLIFYR NHTLRGVFGT MLDSEQARLN
501  PASAVFDSTS RSRITRVSSS STKAAYTTST CFKVVKTNKT YCLSIAEISN
551  TLFGEFRIVP LLVEILKNDG VREARSG
```

15. An antibody which specifically binds an epitope of at least 4 amino acids of a VAP according to claim 11.

16. A host cell, comprising nucleic acid which encodes an antibody according to claim 15.

17. A nucleic acid molecule, corresponding or complementary to at least 15 nucleotides of a DNA sequence encoding at least 5 amino acids of the amino acid sequence of a VAP of claim 11.

18. A nucleic acid molecule according to claim 17, comprising at least 15 nucleotides of Figure 5.

19. A nucleic acid according to claim 18, having the nucleotide sequence of Figure 5.

20. A vector, comprising a nucleic acid molecule according to claim 17.

21. A host, comprising a nucleic acid molecule according to claim 17.

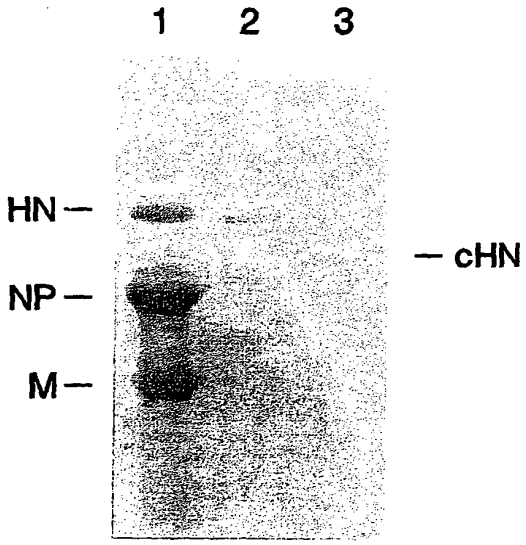

FIG. 1

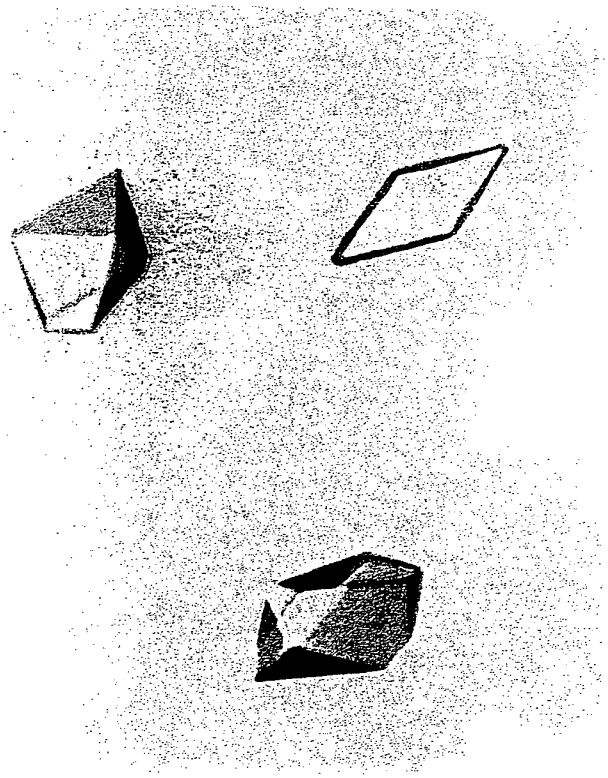

FIG.2

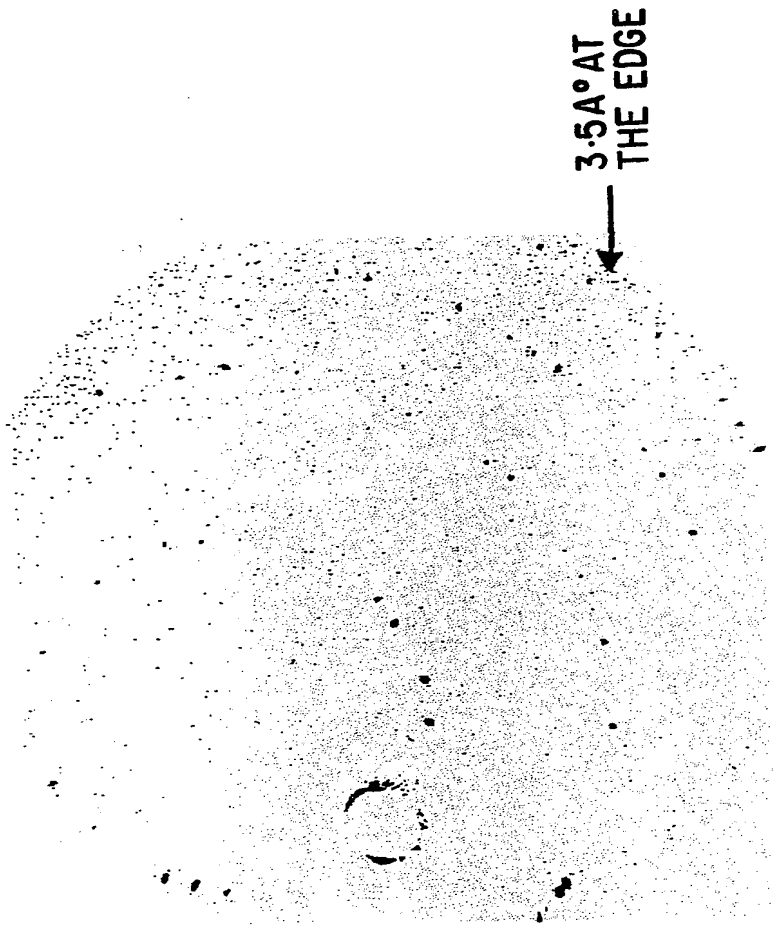

FIG.3

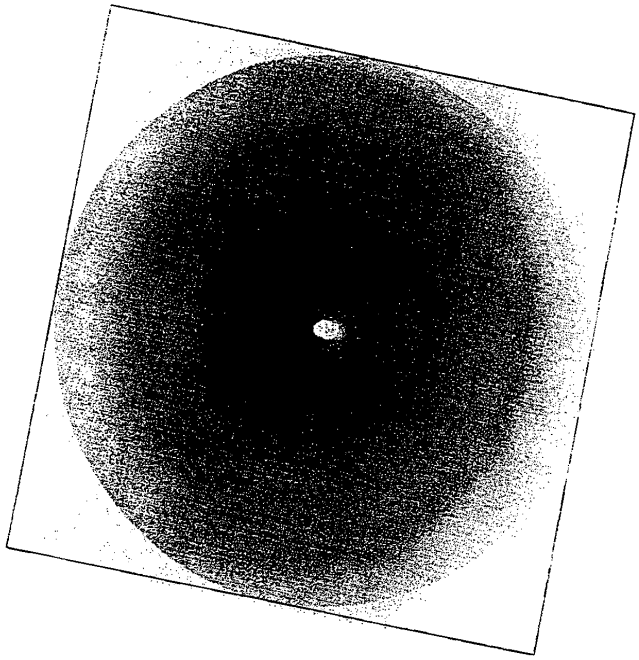

FIG.4

```
   1  ATGGACCGCG CAGTTAGCCA AGTTGCGTTA GAGAATGATG AAAGAGAGGC
  51  AAAAAATACA TGGCGCTTGA TATTCCGGAT TGCAATCTTA CTCTTAACAG
 101  TAGTGACCTT AGCTACATCT GTAGCCTCCC TTGTATATAG CATGGGGGCT
 151  AGCACACCTA GCGACCTTGT AGGCATACCG ACCAGGATTT CTAGGGCAGA
 201  AGAAAAGATT ACATCTGCAC TTGGTTCCAA TCAAGATGTA GTAGATAGGA
 251  TATATAAGCA AGTGGCCCTT GAGTCTCCGT TGGCATTGTT AAACACTGAG
 301  ACCACAATTA TGAACGCAAT AACATCTCTC TCTTATCAGA TTAATGGAGC
 351  TGCGAACAAC AGCGGGTGGG GGGCACCTAT CCATGACCCA GATTTTATCG
 401  GGGGGATAGG CAAAGAACTC GTTGTAGATA ATGCTAGTGA TGTCACATCA
 451  TTCTATCCCT CTGCATTTCA AGAACATCTG AATTTTATCC CGGCGCCTAC
 501  TACAGGATCA GGTTGCACTC GGATACCTTC ATTTGACATG AGTGCTACCC
 551  ATTACTGCTA CACTCATAAT GTAATATTGT CTGGATGCAG AGATCACTCA
 601  CACTCACATC AGTATTTAGC ACTTGGTGTG CTCCGGACAA CTGCAACAGG
 651  GAGGATATTC TTTTCTACTC TGCGTTCCAT CAGTCTGGAT GACACCCAAA
 701  ATCGGAAGTC TTGCAGTGTG AGTGCAACTC CCTTAGGTTG TGATATGCTG
 751  TGCTCGAAAG TCACGGAGAC AGAGGAAGAA GATTATAACT CAGCTGTCCC
 801  TACGCTGATG GCACATGGGA GGTTAGGGTT CGACGGCCAA TACCACGAAA
 851  AGGACCTAGA CGTCACAACA TTATTTGAGG ACTGGGTGGC CAACTACCCA
 901  GGAGTAGGGG GTGGATCTTT TATTGACGGC CGCGTATGGT TCTCAGTCTA
 951  CGGAGGGCTG AAACCCAATT CACCCAGTGA CACTGTACAG GAAGGGAAAT
1001  ACGTAATATA CAAGCGATAC AATGACACAT GCCCAGATGA GCAAGACTAC
1051  CAGATCCGAA TGGCCAAGTC TTCGTATAAG CCCGGGCGGT TTGGTGGGAA
1101  ACGCATACAG CAGGCTATCT TATCTATCAA GGTGTCAACA TCTTTGGGCG
1151  AAGACCCAGC ACTGACTGTA CCGCCCAACA CAGTCACACT CATGGGGGCC
1201  GAAGGAAGAA TTCTCACAGT AGGGACATCT CATTTCTTGT ATCAGCGAGG
1251  GTCATCATAC TTCTCTCCCG CGTTATTATA TCCTATGACA GTCAGCAACA
1301  AAACAGCCAC TCTTCATAGT CCCTATACAT TCAATGCCTT CACTCGGCCA
1351  GGTAGTATCC CTTGCCAGGC TTCAGCAAGA TGCCCCAACT CGTGTGTTAC
1401  TGGAGTCTAT ACAGATCCAT ATCCCCTAAT CTTCTATAGG AACCACACCT
1451  TGCGAGGGGT ATTCGGGACA ATGCTTGATA GTGAACAAGC AAGACTTAAT
1501  CCTGCGTCTG CAGTATTCGA TAGCACATCC CGCAGTCGCA TAACTCGAGT
1551  GAGTTCAAGC AGCACCAAAG CAGCATACAC AACATCAACT TGTTTTAAAG
1601  TTGTCAAGAC CAATAAGACC TATTGTCTCA GCATTGCTGA AATATCTAAT
1651  ACTCTCTTCG GAGAATTCAG AATCGTCCCG TTACTAGTTG AGATCCTCAA
1701  AAATGATGGG GTTAGAGAAG CCAGGTCTGG TTAG
```

FIG.5

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  1  MDRAVSQVAL ENDEREAKNT WRLIFRIAIL LLTVVTLATS VASLVYSMGA
 51  STPSDLVGIP TRISRAEEKI TSALGSNQDV VDRIYKQVAL ESPLALLNTE
101  TTIMNAITSL SYQINGAANN SGWGAPIHDP DFIGGIGKEL VVDNASDVTS
151  FYPSAFQEHL NFIPAPTTGS GCTRIPSFDM SATHYCYTHN VILSGCRDHS
201  HSHQYLALGV LRTTATGRIF FSTLRSISLD DTQNRKSCSV SATPLGCDML
251  CSKVTETEEE DYNSAVPTLM AHGRLGFDGQ YHEKDLDVTT LFEDWVANYP
301  GVGGGSFIDG RVWFSVYGGL KPNSPSDTVQ EGKYVIYKRY NDTCPDEQDY
351  QIRMAKSSYK PGRFGGKRIQ QAILSIKVST SLGEDPALTV PPNTVTLMGA
401  EGRILTVGTS HFLYQRGSSY FSPALLYPMT VSNKTATLHS PYTFNAFTRP
451  GSIPCQASAR CPNSCVTGVY TDPYPLIFYR NHTLRGVFGT MLDSEQARLN
501  PASAVFDSTS RSRITRVSSS STKAAYTTST CFKVVKTNKT YCLSIAEISN
551  TLFGEFRIVP LLVEILKNDG VREARSG
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FIG.6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/14187

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 1/30, 14/25
US CL :530/395, 412

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/186.1, 214.1; 435/235.1, 236; 530/395, 412; 536/23.72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	MURTI et al. Crystals of hemagglutinin-neuraminidase of parainfluenza virus contain triple-stranded helices. Proc. Nat'l. Acad. Sci. February 1993, Vol. 90, pages 1523-1525. See entire document.	1-6, 11 ------- 7-10, 12
Y	THOMPSON et al. Isolation of a biologically active soluble form of the hemagglutinin-neuraminidase protein of Sendai virus. J. Virol. December 1988, Vol. 62, No. 12, pages 4653-4660, especially pages 4653-4654.	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 DECEMBER 1996	Date of mailing of the international search report 23 JAN 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PAUL GOTTLIEB Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/14187

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	TAKIMOTO et al. Crystallization of biologically active hemagglutinin-neuraminidase glycoprotein dimers proteolytically cleaved from human parainfluenza virus type 1. J. Virol. December 1992, Vol. 66, No. 12 pages 7597-7600, see entire document.	1-6, 11 ——— 7-10, 12
Y	SAKAGUCHI et al. Newcastle disease virus evolution: I Multiple lineages defined by sequence variability of the hemagglutinin-neuraminidase gene. Virol. 1989, Vol. 169, pages 260-272, especially page 267.	13,14
Y	WEMERS et al. The hemagglutinin-neuraminidase (HN) gene of Newcastle disease virus strain Italien (ndv Italien): comparison with HNs of other strains and expression by a vaccinia recombinant. Arch Virol. 1987, Vol. 97. pages 101-113. See page 106.	13, 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/14187

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-14

Remark n Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment f additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/14187

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s)1-14, drawn to a method of crystallization of a virus attachment protein (VAP) derived from a membrane of a virus and cleaved by a protease.
Group II, claim(s) 15-16, drawn to an antibody that binds an epitope of a VAP.
Group III, claim(s) 17-21, drawn to nucleic acid molecules encoding hemagglutinin-neuraminidase molecules, the vectors containing these sequences and the host cells containing the vectors.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The inventions of groups I-III are directed to independent and unrelated inventions, i.e., a method for crystallizing a virus attachment protein (Group I), an antibody to a VAP (Group II) and nucleic acids, vectors, and transformed host cells (Group III) and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 as to be directed to a single general inventive concept.